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An ELISA-Like Assay for Hyaluronidase and Hyaluronidase Inhibitors

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Abstract

Hyaluronic acid (HA) is a prominent molecule in the extracellular matrix and is enriched whenever there is rapid tissue proliferation, regeneration and repair. HA is degraded in part by hyaluronidases (HA'ases) that are not well characterized. We have developed a novel ELISA-like rapid assay for HA'ases and their inhibitors. The assay is based on a high affinity biotinylated HA-binding peptide derived from tryptic digests of proteoglycan core protein of bovine nasal cartilage and the avidin-biotin reaction. HA-coated plates were incubated with serial dilutions of *Streptomyces* HA'ase, and the undegraded HA was measured. This established a standard curve for HA'ase activity against which all unknown enzyme samples were compared. The assay is easily modified to also serve a measure of HA'ase inhibitors. For detection of inhibitors, aliquots of sample were preincubated with a known activity of HA'ase and inhibition of HA degradation by the mixture was measured. We have used this assay to document the presence of potent HA'ase inhibitors in fetal calf sera. These techniques will aid in the purification and characterization of Ha'ases and their inhibitors.

Key words: enzyme-linked immunosorbent assay, hyaluronic acid, hyaluronic acid-binding protein, hyaluronidase, hyaluronidase inhibitor.

Introduction

Hyaluronidases (HA'ases) are endoglycosidases that can hydrolyze the N-acetylglucosaminic bonds in hyaluronic acid (HA). Degradation of HA plays an important role in maintaining the integrity of the extracellular matrix. HA'ase and the degradation products of the reaction modulate such important biological processes as wound healing (Bertolami and Donoff, 1978; 1982; Thet et al., 1983), angiogenesis (West et al., 1985) and embryogenesis (Toole and Gross, 1971; Polansky et al., 1973; Belsky and Toole, 1983; Kulyk and Kosher, 1987).

Several assays have been established for the HA'ase group of enzymes. The original assays relied on the reduction of viscosity and turbidity of solutions containing HA (Dorfman, 1948; Dorfman and Ott, 1948) and activity was expressed as viscosity and turbidity reduction units. Currently, HA'ase activity is expressed in National Formulary Units (NFU). The original techniques were useful in establishing sources rich in HA'ases but were relatively cumbersome and insensitive. The HA-impregnated agarose plate assay (Richman and Baer, 1980) is simple, requires only small sample volumes and is suitable for assaying multiple samples simultaneously. However, this technique is not as sensitive as other assays and has therefore not been widely used. More sensitive techniques include a dye-binding assay (Benchetrit et al., 1977) and a recent assay that uses fluorogenic HA as substrate (Nakamura et al., 1990). A zymogram electrophoretic technique using HA-impregnated polyacrylamide (Fiszer-Szafarz, 1984) has the advantage of separating HA'ase isoforms, and HA'ase from its inhibitors, but it is tedious and only semiquantitative. The Reissig assay (Reissig et al., 1955) with a sensitivity of approximately 15 NFU (Linker, 1974), has been the most widely employed HA'ase assay. It is based upon generation of a new reducing N-acetylglucosamine terminus with each

cleavage reaction. Because this assay measures both terminal reducing N-acetylglucosamine and free N-acetylglucosamine, it is sensitive not only to the HA'ases, but also to the combined activities of β-D-glucuronidase and N-acetyl-β-D-hexosaminidase. β-D-glucuronidase attacks the nonreducing terminal glucuronic acid on the HA molecule. N-acetyl-β-D-hexosaminidase may then cleave off the nonreducing terminal N-acetylglucosamine resulting from the action of β-D-glucuronidase. This reaction produces free N-acetylglucosamine that is detected by the Reissig assay. Both exoglycosidases are likely to be present in crude biological preparations, and the use of the Reissig assay may therefore give artifactually high levels of HA'ase due to this activity. β-glucuronidase can be specifically inhibited by including saccharolactone in the reaction mixture (Levvy and Marsh, 1959; Levvy and Conchie, 1966) and substitution of formate for acetate in the buffer results in its partial inhibition (Polansky et al., 1973).

A recent and sensitive HA'ase ELISA based on a brain-derived HA-binding protein and antibodies against that molecule have been described (Delpech et al., 1987). Despite its sensitivity, rapidity and convenience, this ELISA has also not been widely used, presumably due to the need for production and purification of hyaluronectin and anti-hyaluronectin antibodies. We report here a novel ELISA-like assay for HA'ase that is based on a cartilage-derived, biotinylated HA-binding protein and commercially available reagents. The assay can detect 1×10^{-4} NFU of HA'ase and is rapid and simple. Multiple samples can be assayed simultaneously using small volumes of sample. The assay is 1,000 times more sensitive than the widely used Reissig assay. In addition, it can be modified easily to serve as an assay for HA'ase inhibitors. This latter group of substances, though of obvious importance in biological regulation, has not been well defined, presumably because of the lack of a sensitive, reproducible and rapid assay.

Materials and Methods

Production of HA-binding protein

The high affinity HA-binding protein was prepared according to Tengblad (1979), with some modifications, and then biotinylated (Bayer et al., 1979). HA was coupled to AH-Sepharose (Pharmacia, Piscataway, NJ) as described (Tengblad, 1979). Approximately 150 g of bovine nasal cartilage (Pel Freeze, Rogers, AR) was stripped of perichondrium, diced, homogenized in 1 liter of ice cold 4 M guanidine hydrochloride, 0.5 M sodium acetate, pH 5.8, and then extracted for 24 h. This and all other steps in the HA-binding protein preparation were carried out at 4 °C, except where indicated. The extract was passed over a Buchner funnel, without filter paper, to remove the largest cartilage fragments. The filtrate was centrifuged at 6000 g for 30 min and the supernatant collected and dialyzed exhaustively against distilled water using Spectrapor-1 membranes (Spectrum, Los Angeles, CA). A final dialysis was performed against 0.8 mM Tris HCl, 0.8 mM NaCl, pH 8.0. The dialyzed extract was lyophilized and stored at −20 °C.

An aliquot (0.9 g) of lyophilized cartilage extract was rehydrated in 50 cc of buffer containing 0.1 M sodium acetate, 0.1 M Tris-HCl, pH 7.3. Tryptic peptides were generated by incubating with 2.0 mg of trypsin (type III; Sigma, St Louis, MO), at 37 °C. After 2 h, 1.2 mg of soybean trypsin inhibitor (Worthington Chem. Co., Freehold, NJ) was added. This was then placed in dissociative conditions by bringing the extract to a concentration of 4 M guanidine-HCl. This produces a disaggregation of the HA-binding peptide from HA. After 1 h, 70 ml of HA-Sepharose was added and the mixture dialyzed against water in order to "capture" the HA-binding region of the proteoglycan core protein with the HA-Sepharose. To facilitate optimal association of the proteoglycan core protein with the HA-Sepharose, dialysis bags were inverted every 4–6 h to redistribute the settled gel. After exhaustive dialysis, a 70 ml bed volume column was prepared with the HA-Sepharose beads. To remove non-specifically adsorbed material, 300 ml each of 0.5 M sodium acetate with 1 M and 3 M NaCl, pH 5.8, were passed over the column at a flow rate of 26 ml/h. The HA-binding peptides were then eluted from the column with 4 M guanidine-HCl, in 0.5 M sodium acetate, pH 5.8. Fractions of 5 ml were collected, and absorbance at 280 nm was recorded. Fractions containing the protein peak were pooled, concentrated in Centricon 10 tubes (Amicon, Beverly MA) to a final concentration of 1 mg/ml and dialyzed against 0.1 M sodium bicarbonate, pH 8.5. A total of 25 mg of protein was obtained. This HA-binding protein was then biotinylated with biotinyl N-hydroxysuccinimide ester according to the manufacturers instructions (Vector Laboratories, Burlingame, CA), mixed with an equal volume of glycerol and stored at −20 °C.

Assay for hyaluronidase activity

The 96-well microtiter plates (Corning, Corning, NY) were coated with HA obtained from three commercial sources (Sigma, St. Louis, MO; ICN Biochemicals, Costa Mesa, CA; Pharmacia, Piscataway, NJ). This was performed to compare the ability of HA from three different sources to be used as substrates for this assay. The HA was dissolved in water at a concentration of 0.4 mg/ml. This was then diluted in an equal volume of 0.2 M carbonate buffer, pH 9.2. 100-µl aliquots of the diluted HA were applied to each well and the plate and incubated for 16 h at 4 °C.

To establish the ideal concentration of HA for adsorption to the microtiter plates, solutions ranging from 0.1–0.6 mg/ml HA in water were diluted in an equal volume of 0.2 M sodium carbonate buffer, pH 9.2. 100-µl

aliquots of the diluted HA were applied to each well. The plates were then incubated for 16 hours at 4 °C and adsorbed HA detected as described below.

All incubations were followed by three rinses in PBS containing 0.05% Tween 20 (Fisher Scientific, Fair Lawn, NJ). The wells were exposed to the wash buffer for approximately 15 s. All assays were performed in triplicate. Values represent the mean of triplicate wells; bars indicate the standard deviation.

To establish concentration-dependence for the assay, the HA-coated wells were incubated with 100-µl aliquots for 5 h at 37 °C with serial dilutions of *Streptomyces* HA'ase (Calbiochem, San Diego, CA) in 0.1 M sodium acetate, 0.15 M NaCl, 0.2 mg/ml BSA, pH 5.0. To establish time-dependence, wells were incubated at 37 °C with 100-µl aliquots of 1×10^{-3} NFU HA'ase for periods between 1 and 20 h.

Following the HA'ase incubation, non-specific binding by subsequent reagents was blocked by incubating with 300 µl/well of ELISA blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 min at 37 °C. Alternatively, incubation with blocking reagent could be carried out prior to HA'ase digestion.

The HA remaining after digestion was detected using the biotinylated HA-binding protein. To establish the ideal concentration of the HA-binding protein for detecting adsorbed HA, the protein was serially diluted in a buffer of 25 mM sodium phosphate, 0.15 M NaCl, 0.3 M guanidine-HCl, 0.08% bovine serum albumin, and 0.02% sodium azide, pH 7.0. 100 µl of the diluted HA-binding protein was then applied to each well and the plate was incubated for 1 h at 37 °C. Next, to amplify the signal of biotinylation, wells were incubated with anti-keratan sulfate monoclonal antibody (ICN Biochemicals, Costa Mesa, CA) (1:1000 in PBS) for 30 min at room temperature. This was followed by incubating with biotinylated anti-mouse Ig (Vector, Burlingame, CA) (1:200 in PBS) for 30 min at room temperature. The biotinylated complex was detected with the avidin-biotin-peroxidase complex coupled to a reaction using *o*-phenylenediamine as a substrate as described by the manufacturer (Vector, Burlingame, CA). Absorbance was read at 492 nm.

Streptomyces HA'ase was diluted in 0.1 M sodium acetate, 0.15 M NaCl, 0.2 mg/ml BSA. Liver HA'ase was prepared as described (Stern and Stern, 1990) and diluted in 0.1 M sodium formate, 0.15 M NaCl, 0.2 mg/ml BSA. To determine pH activity profiles, the pH of each enzyme was adjusted with acetic and formic acids, respectively. Protein concentrations were assayed using the BioRad protein dye kit with BSA used as a standard.

To compare the sensitivity of the ELISA-like assay with an established method, we utilized the Reissig assay (1955). HA'ase activity was determined by measurement of terminal N-acetylglucosamine released during incubation of HA with dilutions of *Streptomyces* HA'ase. 5-µl aliquots of HA'ase were incubated at 37 °C for 16 h with 0.5 cc of 1 mg/ml HA and released N-acetylglucosamine measured colorimetrically as described.

Assay for HA'ase inhibitors in fetal calf serum

The HA'ase assay was easily modified to serve as an assay for HA'ase inhibitors. Serial dilutions of fetal calf serum which is a potent source of inhibitor were made in PBS. 1×10^{-3} U/ml of *Streptomyces* or partially purified liver HA'ase was then mixed with an equal volume of the serially diluted fetal calf serum or PBS. These mixtures of enzyme and inhibitor were incubated for 1 h at 37 °C prior to application to the microtiter plate. During this incubation, inhibition of the enzyme by FCS occurred. 100-µl aliquots of these mixtures were then applied to HA coated wells and assayed for HA'ase activity as described above. The percent inhibition was calculated as $\%I = 1 - [(A_{max} - A_{sample}) / (A_{max} - A_{min})]$ where A_{max} is the absorbance of wells not exposed to HA'ase, A_{min} is the absorbance of wells exposed to HA'ase plus an equal volume of PBS (no inhibitor), and A_{sample} is the absorbance of wells exposed to HA'ase plus an equal volume of sample containing inhibitor. Percent inhibition is thus determined from differences between the absence of HA degradation, HA degradation produced by a known enzymatic activity, and the degradation produced by a known enzyme activity exposed to enzyme inhibitors.

Results

An idealized representation of the ELISA-like assay is presented in Figure 1. The proteoglycan core protein of cartilage from which the HA-binding protein is derived functions like an antibody to HA. The binding protein contains keratan sulfate glycosaminoglycan chains covalently attached to the core protein. We took advantage of this and amplified the signal of biotinylation on the HA-binding protein by exposing the HA-binding protein to an anti-keratan sulfate monoclonal antibody and a secondary biotinylated antibody (Fig. 1). Inclusion of the anti keratan sulfate antibody and biotinylated secondary antibody approximately doubled the sensitivity of the assay.

The HA preparations from Sigma, ICN and Pharmacia were examined for use in this assay. Solutions of 0.4 mg/ml were diluted 1:2 in 0.2 M sodium carbonate buffer, pH 9.2 and incubated in the microtiter plate for 16 h at 4 °C. The assay was run without exposure to HA'ase. HA from Sigma gave the highest level of responsiveness. This preparation of HA was therefore used as the substrate for all subsequent experiments.

To determine the optimal concentration of HA needed to coat the microtiter plates, solutions of Sigma HA was prepared ranging from 100–600 µg/ml in water and diluted 1:2 in sodium carbonate buffer to a final concentration

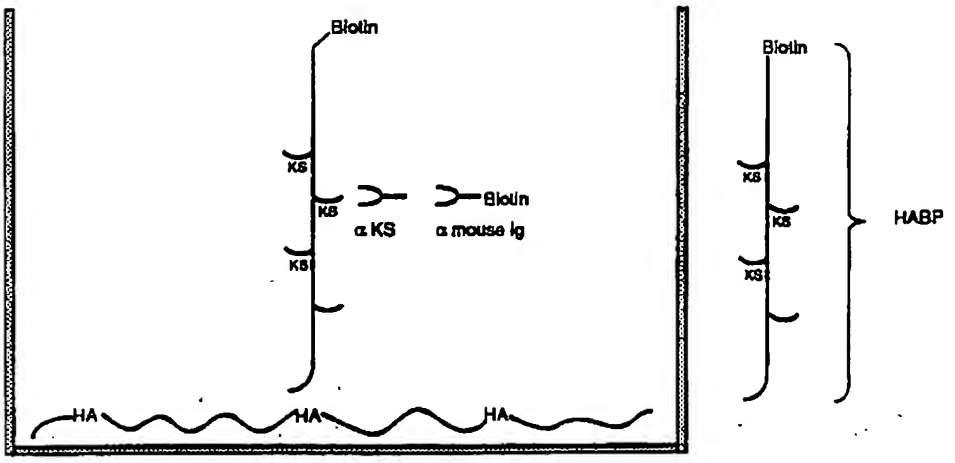

Fig. 1. Schematic diagram of hyaluronidase ELISA. Hyaluronic acid (HA) after HA'ase digestion was detected with the biotinylated HA-binding protein (HABP). The biotin signal was amplified by incubating with monoclonal antibody against keratan sulfate (αKS) and a secondary, biotinylated antibody. Bound complex was detected with the avidin-biotin-peroxidase technique.

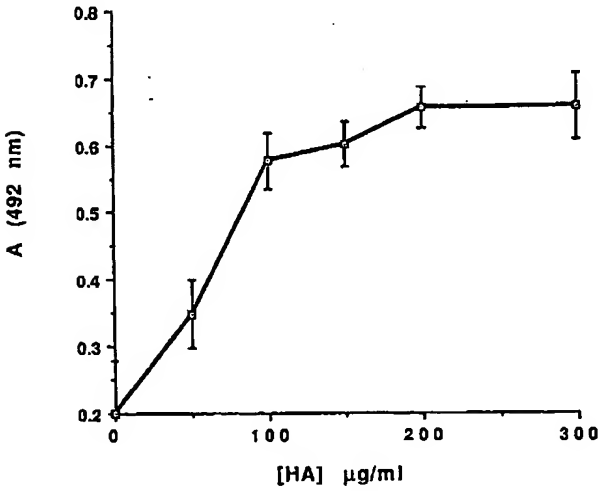

Fig. 2. Effect of HA concentration on responsiveness of the assay. Various concentrations of Sigma HA were diluted in water. These solutions were then diluted (1:2) in carbonate buffer and adsorbed to wells as described in Material and Methods. [HA] values represent the HA concentration after dilution in carbonate buffer. In this and subsequent figures, values are means of triplicate wells; bars indicate standard deviation.

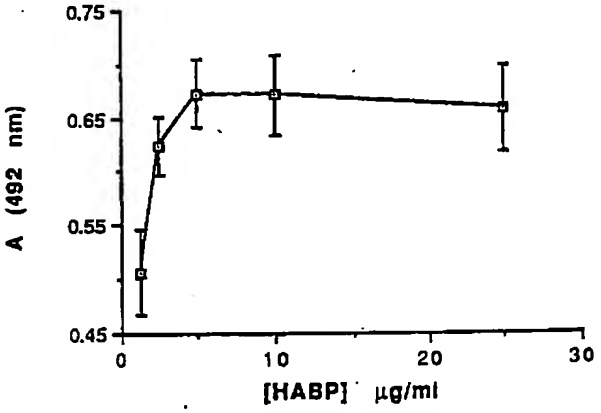

Fig. 3. Effect of HA-binding protein concentration on the detection of adsorbed HA. To determine the minimal effective concentration of HA-binding protein needed for this ELISA, HA-binding protein was diluted in its buffer and used to detect adsorbed HA as described in Materials and Methods.

between 50–300 µg/ml. The optimal concentration of HA for coating was 200 µg/ml after dilution in carbonate buffer (Fig. 2). This concentration was therefore used to coat the wells in all subsequent experiments.

The ideal concentration of the biotinylated HA-binding protein was determined by serial dilutions of the reagent in buffer. Again, the assay was performed without exposure to HA'ase to determine the maximal absorbance. Maximal absorbance was obtained when the HA-binding protein was diluted to a protein concentration of 5 µg/ml (Fig. 3). This concentration of the HA-binding protein was therefore used in all subsequent experiments.

Enzyme assays must be both time- and dose-dependent to be valid. Such dependency is seen in Figures 4 and 5. HA degradation plateaus between 5 and 6 hours. The degradation is linear between 10^{-3} and 10^{-4} NFU/ml HA'ase.

The degree of sensitivity of the ELISA-like assay is 1000 times greater than the Reissig assay. Figure 6 demonstrates a dose dependent decrease in N-acetylglucosamine released by dilutions of *Streptomyces* HA'ase. The limit of sensitivity of the Reissig assay in our hands is 0.1 NFU/ml HA'ase. A sensitivity of 15 NFU is reported by Linker (1974).

Similar enzymatic activities from different sources can occasionally be distinguished by their pH profiles (Gold,

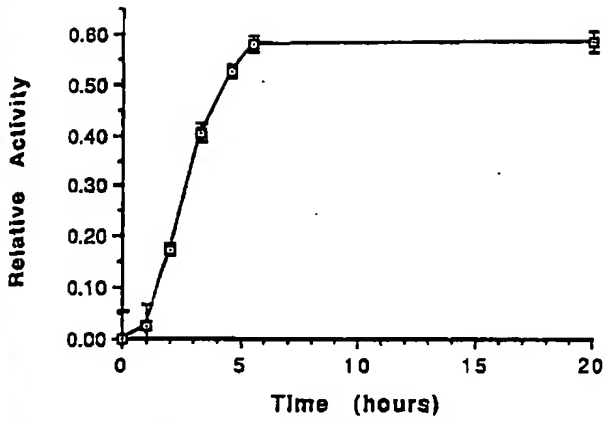

Fig. 4. Time-dependency of *Streptomyces* hyaluronidase enzyme activity. Hyaluronidase was added to HA-coated microtiter plates at a concentration of 1×10^{-3} NFU and incubated for various times. Relative activity represents the difference in absorbance between wells not exposed to HA'ase and those exposed to this enzyme.

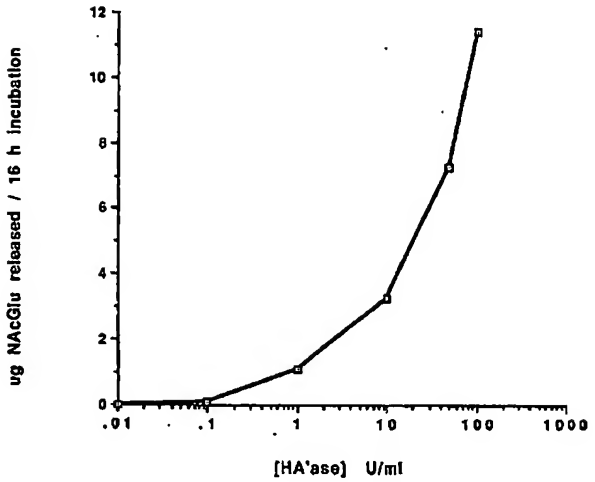

Fig. 6. Sensitivity of the Reissig assay. Dilutions of *Streptomyces* HA'ase were incubated with HA for 16 hours and released terminal N-acetylglucosamine measured as described. The assay can detect no less than 0.1 NFU/ml HA'ase.

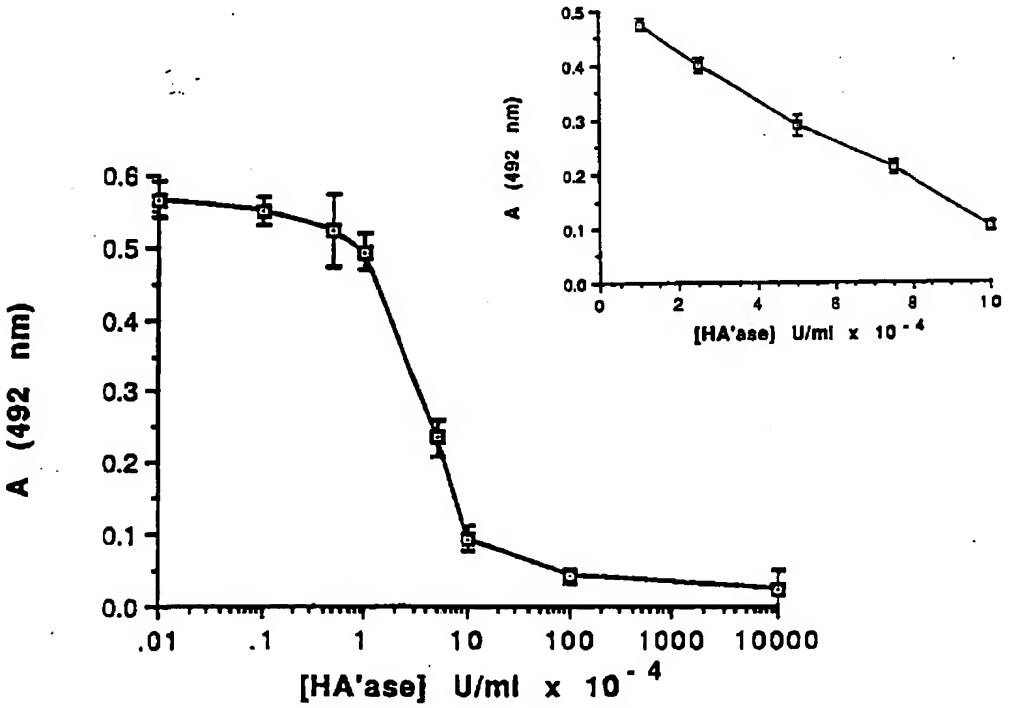

Fig. 5. Dose-dependency of *Streptomyces* hyaluronidase enzyme activity. Serial dilutions of hyaluronidase were incubated with HA-coated microtiter plates for 5 hours at 37 °C as described in Materials and Methods. The reaction is linear between 10^{-3} and 10^{-4} U/ml (inset).

1982). We compared the activity at various pH's of *Streptomyces* HA'ase and a partially purified porcine liver HA'ase (Stern and Stern, 1990). Enzymes were diluted in buffer at the indicated pH's and assayed for activity (Fig. 7). *Streptomyces* HA'ase was most active at pH 5 and had a broad activity profile whereas the porcine liver HA'ase had optimal activity at pH 4. The pH optima for the HA'ases found in porcine kidney extracts and the urine of children with Wilms' tumor are 3.5 (Stern et al., 1991).

By modifying the HA'ase assay to serve as a measure of HA'ase inhibitors, we were able to detect potent HA'ase inhibitors in fetal calf serum. Both *Streptomyces* and liver

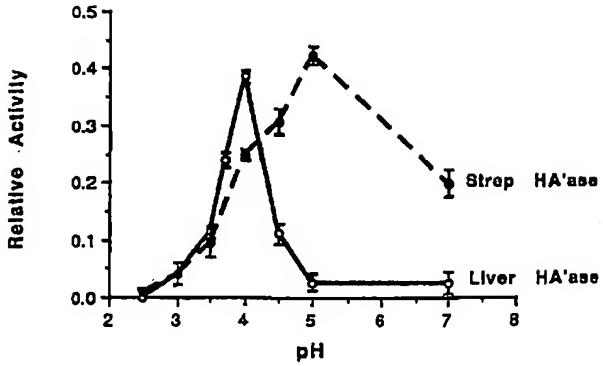

Fig. 7. Activity of liver and *Streptomyces* HA'ases as a function of pH. The *Streptomyces* enzyme was prepared in a buffer containing 0.1 M sodium acetate, 0.15 M NaCl 0.2 mg/ml BSA at the pHs indicated. The liver HA'ase was prepared in a similar buffer containing sodium formate in place of sodium acetate. Relative activity represents the difference in absorbance between wells not exposed to HA'ase and those exposed to this enzyme.

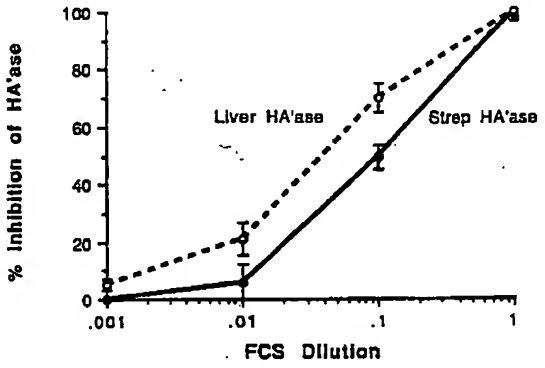

Fig. 8. FCS contains inhibitors of *Streptomyces* and liver HA'ases. FCS was serially diluted in PBS and incubated with an equal volume of either *Streptomyces* or porcine liver HA'ase. Resulting enzymatic activity was then assayed as described in Materials and Methods and activity compared to that in enzyme samples that had been incubated with PBS.

HA'ases were inhibited by fetal calf serum in a dose dependent fashion (Fig. 8). Approximately 50% and 70% inhibition was obtained by adding an equal volume of a 1:10 dilution of FCS to 1×10^{-3} U/ml of *Streptomyces* and liver HA'ase respectively.

Discussion

We describe here a rapid and convenient ELISA-like assay for the detection of HA'ase and with minor modifications, an assay for HA'ase inhibitors. Multiple samples can be analyzed in less than 9 h. Since each well of the microtiter plate is incubated with 100 µl of sample, the total sample volume, tested in triplicate, requires no more than 300 µl. The low sample volume and ability to assay multiple fractions make this assay particularly suitable for protein purification and for determination of activity in small biological samples. The assay can detect as little as 1×10^{-4} NFU of activity. This sensitivity is greater than that of any assay previously reported with the exception of the assay described by Delpech (1987). The advantage of the assay reported here is that all reagents are commercially available or can be conveniently prepared. The HA-binding protein is easily purified and is a highly stable reagent. When stored at −20 °C the binding protein retains its full activity for at least 2.5 years. A second advantage of this assay is that it can be modified to serve as an assay for HA'ase inhibitors. Serum contains HA'ase inhibitors and may play a role in tumor progression (Fiszer-Szafarz, 1968). However, these inhibitors have not been isolated or characterized, primarily because a rapid and convenient assay has heretofore been lacking.

Since this assay uses substrate that is in solid phase, accurate determination of HA adsorption to the plate can not be made. This presents difficulties in calculations of enzyme kinetics. In addition, cleavage of the HA polymer by HA'ase produces "nicks" in the polymer that may not necessarily result in displacement from the microtiter plate. We postulated that the negatively charged, hydrophilic HA adsorbs to the negatively charged hydrophobic polystyrene plate primarily via HA-associated proteins. This suggests that internal cleavage of the polymer may produce oligosaccharide chains that are protein-free and therefore released from the plate. Regardless of the actual mechanism of HA displacement from the plate following exposure to HA'ase, the assay is time- and dose-dependent.

Regulation of HA metabolism in developing tissues, healing wounds, and the stroma of malignant tumors plays a critical role in these cellular processes. It is likely that regulation involves a balance between factors that stimulate HA synthesis (Decker et al., 1989) and factors that regulate its degradation. This degradation may in turn involve a balance between HA'ase and HA'ase inhibitors. Based on the information derived from the turnover of other extracellular matrix molecules, we predict the existence of several classes of physiologically relevant HA'ase inhibitors. The ELISA-like assay described here will facilitate the purification and characterization of these important molecules.

Acknowledgements

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